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PHENOLIC COMPOUNDS OF THE WHEAT PLANT IN RELATION TO RUST RESISTANCE

By John Ansel Anderson

Department of Field Crops

University of Alberta,
Edmonton, Alberta.
April, 1928.

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By John Ansel Anderson
Department of Field Crops

A THESIS

submitted to the University of Alberta
in partial fulfilment of the requirements for
the degree of

MASTER OF SCIENCE.

Edmonton, Alberta.
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PHENOLIC COMPOUNDS OF THE WHEAT PLANT IN RELATION TO RUST RESISTANCE

By John Ansel Anderson

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INTRODUCTION.

Because of the immense economic losses due to the ravages of Wheat Stem Rust, the Research Council of Canada innitiated an extensive program of research on this disease, with the hope of finding methods of control. Biochemical investigations on the nature of susceptibility and resistance were undertaken at the University of Alberta, under the direction of Dr. R. Newton. Part of the program outlined consisted of the analysis of the tissue of wheat varieties of graded resistance to rust, for organic compounds which might play a part in the physiology of rust, either as substances toxic to the fungus or as essential foods. For reasons which are set forth below, the phenolic compounds were chosen for first investigation. The progress which has been made in these studies is reported in this thesis.

[illegible] OF THE [illegible] POLICY
OF [illegible] OF [illegible] [illegible]

by John James [illegible]

[illegible]

[illegible] of the [illegible] [illegible] in the [illegible] of [illegible] [illegible], the [illegible] [illegible] a [illegible] [illegible] [illegible]

THE PHENOL HYPOTHESIS.

The various hypotheses which have been put forward to explain the infection capabilities of the different biologic forms of the rust fungus, Puccinia graminis tritici, and the degrees of resistance of the different wheat varieties, are based on knowledge of the behaviour of the fungus in resistant and susceptible hosts. This knowledge has been obtained by cytological investigations.

In 1902 Ward started cytological investigations of two species of Puccinia. In his early work on P. dispersa (48) he realised that structural features elucidated by the microscope were not responsible for the phenomena of immunity and susceptibility on the part of the host, or of capacity or incapacity to infect on the part of the fungus, and he therefore concluded that the phenomena resided in the individual properties of the living cells. Two explanations were put forward: (49, p. 139) "The fungus, when growing on a species of Bromus A, may refuse to infect another species B, either because B secretes some body of the nature of an enzyme or anti-toxin which effectually keeps the mycelium of the fungus at bay, or because the fungus habituated to the peculiar nutritive or other conditions afforded it by the host-plant A, cannot immediately adapt itself to the very different conditions offered by the species B." After investigations of the nutritive theory it was discarded (49). In a later paper (50, p. 21)

Ward sums up his general conclusions as follows: "In other words, infection, and resistance to infection, depend on the power of the Fungus-protoplasm to overcome the resistance of the cells of the host by means of enzymes or toxins; and, reciprocally, on that of the protoplasm of the cells of the host to form anti-bodies which destroy such enzymes or toxins, or to excrete chemotactic substances which repel or attract the Fungus-protoplasm." In the same paper (p. 39), in discussing the behaviour of P. glumarum on immune wheat varieties, a further suggestion is put forward: "The only conclusion we can come to is that the hyphae attack the cells too vigorously at the outset; instead of capturing them, as it were, by putting in haustoria which delicately tap the cell-contents and make them serve as sources of food-supplies, the Fungus clumsily kills these cells, and is in consequence subjected to all the exigencies of starvation, or worse". The hypotheses of Ward are given in detail since in spite of many investigations of a similar nature, some of which are cited below, they still remain unchallenged.

Gibson (15), a student of Ward, studied the behaviour of several species of rust fungi in plants unrelated to their natural hosts. She rejects the idea of starvation and attributes the death of the fungus after entrance to toxic substances emitted by the host cells. Marryat (24), another student of Ward, worked with Puccinia glumarum on wheat. She interprets her results as evidence in favour of the toxin-antitoxin explanation of resistance and susceptibility.

Stakman (38) studied the behaviour of Puccinia graminis tritici on susceptible and immune wheat varieties. He concludes that immunity is due to "physiological incompatibility as evidenced by the killing of the host cells by the fungus and the more or less sudden death of the fungus itself". A general statement such as this covers all the hypotheses.

Newton (26) favours the third explanation, that the failure of the rust fungus to infect resistant hosts is due to the starvation of the parasite by the local killing of the host cells.

Wellensiek (51) working with Puccinia sorghi, emphasizes the fact that the differences in development on resistant and susceptible hosts are only quantitative. He accounts for these differences by assuming that a special nutritive substance is present in varying amounts in different varieties of the host plant. The killing of the host cells which occured in resistant varieties he attributes to toxic substances excreted by the fungus after its death from starvation.

The most comprehensive and detailed cytological investigation of the behaviour of Puccinia graminis tritici in susceptible and resistant wheat varieties has been made by Allen (1, 2, 3). Definite evidence is brought forward to show that the fungus secretes some substance into the host cell. In susceptible hosts this substance stimulates the cell and it produces additional food to meet the demands of the fungus. In resistant hosts the first cells attacked by the fungus are killed

outright, apparently as a result of the fungal secretion. Cells which are attacked after the initial attempt show progressively less violent reactions and the fungus is often able to establish itself and continue to grow for some time. Evidence is brought forward in support of the suggestion that different biologic forms of rust differ in their secretions and that the chemical response of the host varies according to the secretion it receives. In general, Allen concludes that resistance and susceptibility to rust may be explained by differences in the response of the host cell protoplasm to substances secreted by the fungus.

After due consideration of the evidence brought forward in the papers reviewed above, the toxic substance hypothesis appears to offer the most plausible explanation of rust resistance in wheat. The terms toxin and antitoxin have been used by other workers; but because of their connotation it seems inadvisable to use them in plant pathology. In animal pathology toxin refers to soluble poisonous products of bacterial growth or decay, and antitoxin refers to those products of the blood sera which are produced on introduction of the toxin and which have the power of counteracting its effect. There is little evidence to show that such a mechanism occurs in plants, at least in connection with fungous diseases. It is true that the rust fungus probably excretes some substance into the host cell, but whereas in animal diseases the bacterial toxin is injurious to the cells of both immune and susceptible individuals, such is

not the case with rust. Here we find that the cell of a susceptible host is not injured by the fungal excretion but is rather stimulated to more active metabolism. If the antitoxin hypothesis is retained, these facts must be explained by postulating that the fungal excretion is a toxin, but that the cell immediately counteracts its effect by the production of an antitoxin. In resistant hosts on the other hand, the substance excreted by the fungus is apparently fatal to the cell, and the cell contents in their turn inhibit the growth of the fungus. According to the antitoxin hypothesis, the cell is killed because it does not produce an antitoxin. The death of the fungus may then be attributed to starvation brought about by its own action in killing the cell and thus preventing it from producing the necessary food. These explanations, however, strain the meanings of the words toxin and antitoxin considerably. They also entail the paradoxical statement that susceptibility is extreme resistance, and vice versa, that resistance is extreme susceptibility.

By dropping the toxin-antitoxin hypothesis a much simpler and more plausible explanation can be offered. The substance excreted by the fungus probably contains enzymes whose function is to modify the contents of the host cell so that they can be absorbed as foods by the fungus. In susceptible varieties the enzymes fulfil their function without apparent damage to the cell. In resistant varieties, the action of the enzymes instead of producing foods, or besides producing them, sets free some substance in the host cell which is toxic to both the

fungus and the cell itself. This hypothesis therefore postulates that there are in resistant varieties substances which are potentially toxic to the fungus.

A review of the literature on disease resistance offers certain indications of the class of compounds which may play a part in resistance as toxic substances. Cook and Taubenhaus (7, 8) found cases in which disease resistance was apparently correlated with the rate of tannin formation in the host cells. Working with sixty-six organisms they also found that tannic acid was more toxic than a variety of other vegetable acids, and that in general it was more toxic to parasitic than to saprophytic fungi. Cook and Wilson (9) working with members of the genus Endothia in cultural media found that in most cases growth was retarded by 0.8% of tannin. Endothia parasitica however, was able to use tannin as a food. In general they claim that tannin inhibits fungal growth and that resistance in certain plants to attack by fungous diseases is due to tannin in their cells as one factor. Graves (17) investigated the cause of persistent development of vigorous basal shoots from the roots of chestnut trees which had been killed by blight. The parasite was found to grow more slowly in the roots than in the stems and it is suggested that this is due to the fact that the roots contain twice as much tannin as the stems.

Hawley, Fleck and Richards (21) found a high correlation between the keeping quality of wood and the toxicity of water extracts of the wood to fungi. The toxic properties of

the extracts are attributed to tannins and water-soluble colouring matter of unknown composition.

Walker (44) investigated smudge in onions. Coloured varieties were found to be resistant as a class whereas colourless varieties were susceptible. The pigments are confined to the dry outer scales. The author offers evidence showing that the chief factor causing resistance to smudge is apparently a substance closely associated, if not identical with these pigments. Their functioning, according to Walker and Lindengren (46) depends on the ready diffusability of these pigments out of the dead tissue into the soil water where they inactivate the fungus before attack. The press-juice of the inner scales of the onion was also toxic to fungi but no difference existed between the toxicity of extracts of resistant and susceptible varieties. The authors therefore conclude that the cell sap is not a factor in resistance but may be important in limiting growth after the fungus has entered. Investigating the nature of these toxic substances of the juice, Walker et al (47) found them to be of two types, one of which is neither removed nor readily broken down by heat and one of which is readily volatile. In later work with neck rots of onion, Walker (45) offers the same explanation of resistance.

Tins (42) suggests that certain varieties of cabbage owe their resistance to cabbage yellows to a greater content of sulfur-containing compounds, but offers no experimental evidence on this point. Reynolds (32) attributes resistance to wilt in certain varieties of flax to a higher content of the glucoside

linamarin, which can be split by the enzyme phaseolutinase to produce free hydrocyanic acid.

Of the various substances mentioned above, those most likely to occur in wheat are the water-soluble pigments, related compounds, and tannins. A consideration of the structural formulae of these two classes of compounds shows that they are related in that the molecules of both contain various simple phenols. This suggests that their toxicity may be due to their phenolic properties, since it is well known that many of the simple phenols have antiseptic and germicidal properties. With these considerations in mind a tentative hypothesis which would form the basis of an experimental investigation was drawn up as follows: resistance to stem rust in wheat is due to phenolic compounds set free in the cell on the entrance of the fungus; these kill the cell and inhibit the growth of the parasite.

A review of the literature on the antiseptic, germicidal and fungicidal properties of phenolic compounds has yielded information which serves to substantiate the phenol hypothesis by offering further suggestions as to the part these compounds may play in rust resistance.

Small differences in the structure and composition of phenolic compounds have been shown to effect their antiseptic and germicidal efficiency considerably. Johnson and Lane (22) find that the incorporation of an alkyl group in the aromatic nucleus of resorcinol increases its antiseptic properties, and that the increase is a function of the size of the group introduced. Tilley and Schaffer (41) find that the same holds true

[illegible]

[illegible]

[illegible]

for the germicidal activity of phenol. Similarly, in a survey of phenolic fungicides, Gray (18) reports that cresols are more effective than phenol. This suggests that small differences in the composition of phenolic compounds occuring in different varieties of wheat might have marked effects on their resistance to various rust forms.

Cooper and Mason (10) find that B. fluorescens non-liquifaciens is selectively attacked by physico-chemical germicides such as phenol, whilst B. coli is less sensitive to them more sensitive to chemical germicides such as the quinones. Substitution of various groups in the aromatic nucleus affects the germicidal power on these two bacteria unequally, with the result that the selective action may be obscured or even reversed. These facts suggest that the different infection capabilities of the various biologic forms of rust may be due on the one hand to small differences in the composition of phenolic compounds occuring in different wheat varieties, and on the other, to differences in the toxicity of a given phenol to the different biologic forms of the fungus.

In putting forward these suggestions it has been taken for granted that differences in composition and structure of phenols will have similar effects on fungicidal activity to those on antiseptic and germicidal activity. Justification of this supposition is based on the assumption that the fungicidal properties of phenols, like their antiseptic and germicidal properties, depend on their ability to coagulate proteins.

The hypothesis, that the fungal excretion sets free in the cells of resistant hosts a substance which is toxic to both the fungus and the cell, may be elaborated along other lines. If the substance produced is responsible for the death of the cell by denaturation or coagulation of the cell proteins it can not exist as such in the cell under normal conditions. It seems probable that it would be present in some non-toxic combination or form, rather than that it would be synthesized only on the entrance of the fungus. If it be postulated that the toxic substance is a relatively simple phenol, it may occur in the cell as a constituent of the molecule of a more complex substance such as a pigment or tannin; or again, if the toxic substance be one of the more complex compounds, they may occur in innocuous form as glucosides. The action of the fungal secretion postulated in resistant hosts results in the breaking down of the complex substances which sets free the toxic compounds. The possibility of the enzymes secreted by the various rust forms differing in their ability to bring about this decomposition, or of different compounds in the various wheat varieties differing in their resistance to such decomposition, offers further possible explanations of the mechanism of differential resistance.

It seems almost too much to hope that the relatively simple suggestions put forward above will serve to explain all the complications of rust reactions in wheat. However a more specific working hypothesis than those based on cytological investigations was absolutely essential in order to narrow the field of investigation, and since none more plausible suggested

themselves, the phenol hypothesis was tentatively adopted.

INVESTIGATION OF PHENOLIC COMPOUNDS OF THE WHEAT PLANT.

QUANTITATIVE ANALYSIS.

Comparisons of the quantities of phenolic compounds occuring in susceptible and resistant wheat varieties offer one method of testing the truth of the phenol hypothesis. The program drawn up for this investigation included the analysis of the tissue and press-juice of eight wheat varieties of graded resistance to rust, which have been used in previous studies on rust resistance.

We have so far been unable to find any definite information in the literature on the phenolic compounds occuring in wheat plants. Tannins are almost universally present in plants. Shibata, Nagai and Kishida (37) find that flavone derivatives are very widely distributed and they may therefore be expected to occur in wheat. Collison (6) working on the toxicity of extracts of plant residues to the growth of seedlings, obtained qualitative tests for vanillin in extracts from wheat straw, and tests for vanillin and salicylic acid in extracts of alfalfa hay. (It is interesting to note that he suggests that some of these rare substances with antiseptic properties may play a part in the prevention and limitation of fungal attack.) Ignorance of the phenolic compounds present in the wheat plant, and the probability of such compounds occuring in minute amounts,

complicate the investigation.

Two general methods of procedure suggested themselves: (1) to make a qualitative investigation of the phenolic compounds occuring in the wheat and to follow this with an attempt to develop quantitative methods for comparing varieties; and (2) to make a general quantitative survey of the phenol content of the varieties, and if this showed marked differences favouring the hypothesis, to make a more detailed investigation later. Since the latter method offered a chance of arriving at a conclusion with regard to the soundness of the hypothesis more quickly, it was adopted in preference to the longer but more logical method of investigation.

Methods for the Determination of Phenols.

Gibbs (14) has reviewed the literature on phenol tests and has classified them according to the chemistry of the reaction involved. More than forty tests varying in the character of the reagent are reported, and these have been so modified and complicated in various ways that the tests described in the literature number more than a hundred. A few of these tests have been applied on a quantitative basis. The large majority are not susceptible to quantitative application, and are not qualitatively specific for any phenol, and some are not specific for phenols as a class but are given by other substances not phenols.

Since quantitative determination of phenols have been made on such complex physiological fluids as blood (4, 28, 31, 39)

and urine (12, 43), their appeared to be no a priori reason why one of the methods should not be adapted to the determination of phenols in the press-juice from wheat leaves. The older volumetric methods have been discarded in favour of colorimetric methods. The colour reagent of Folin and Denis (11) has been used almost exclusively. It is a phosphotungstic-phosphomolybdic complex which is reduced by phenols to give a bright blue colour, The reagent is not specific for phenols. Gortner and Holm (16) have pointed out that it reacts with a number of protein degradation products and Levine (23) finds that it gives a colour with a great variety of compounds and concludes that, in general, it reacts with all sorts of compounds having more or less reducing properties.

Hanke and Koessler (19) developed a quantitative colorimetric method for mixtures of certain phenols. It depends on the formation of a soluble azo dye by the reaction of diazotized sulfanilic acid with the phenol in alkaline solution. The reagent has certain disadvantages. It produces different colours with different classes of phenols. Thus with phenols in which the para position is unoccupied it gives a yellow; with phenols in which the para position is occupied by a substitute containing no amine group it gives a red. Where mixtures of different members of the two classes occured it might therefore be impossible to use the reagent owing to the difficulty of making quantitative colorimetric comparisons of two liquids which contain two colours mixed in different proportions. The conditions under which

accurate quantitative measurements could be made were also found to differ from one phenol to another. The reagent is not specific for phenols; it reacts with aromatic compounds in which an amine group replaces the hydroxyl group of the phenols, with ammonia and amines, with various compounds containing a ketone group and with certain alcohols. However, in spite of these objections, Theis and Benedict (39) have obtained satisfactory results for phenols in blood by the use of a similar reagent, diazotized p-nitroaniline.

Since neither of these reagents is specific for phenols as a class the problem of determining phenols by their use is largely one of getting rid of interfering substances. In the recent work on blood and urine cited above, the most important interfering substances have been precipitated by the use of various reagents. In earlier work on urine, reviewed by Tisdall (43), several methods were developed for the separation of volatile phenols by steam distillation. Extraction of the phenols from acid solution with ether, followed by extraction from ether with dilute sodium hydroxide solution has been used by Tisdall (43) and by Hanke and Koessler (20).

Since lack of knowledge of the phenols and interfering substances which occur in wheat prevented the planning of a detailed investigation and method, the following broad tentative program was drawn up.

Tentative Program of Investigations.

Quantitative analysis of tissue and press-juice of wheat plants.

A. Determination of uncombined phenols.

1. By steam distillation of the press-juice.

2. By ether extraction of the press-juice and tissue.

B. Determination of phenols in simple combination by acid hydrolysis of the press-juice and:

1. Steam distillation.

2. Ether extraction.

C. Determination of compounds containing phenolic groups.

1. Tyrosine.

2. Pigments - Flavone, Flavanol, Anthocyanidin.

3. Tannins.

Qualitative analysis of tissue.

EXPERIMENTAL

Definitions

Since we have no knowledge of the phenols which occur in wheat, the term "phenol" must be interpreted throughout this report in conjunction with the method of extraction and determination. Thus, "volatile phenols" refers to those compounds which are volatile with steam and which react with the phenol reagent used. Again, "total phenols" refers to those compounds which are extracted by ether from the filtrate from press-juice after precipitation of the proteins by tungstic acid, re-extracted from the ether by sodium hydroxide solution, and which give a blue colour with the Folin-Denis phenol reagent.

Qualitative Problems in Interpretations.

Qualitative research on Islam has been [illegible] in three ways.

1. Mathematization or quantitative research.

   1. By means [illegible] of the [illegible].

   2. By [illegible] of the [illegible].

2. Formalization of semantics [illegible] of the semantics of the [illegible]:

   1. Tense classification.

   2. Tense motivation.

3. Interpretation of [illegible] relations [illegible].

   1. Tendency.

   2. Simple - Tense, Tense, [illegible].

   3. Tense.

Qualitative research is [illegible].

Conclusion

Allow me here to emphasize [illegible] the [illegible] [illegible] - [illegible] [illegible] [illegible]

Attempt to Determine Volatile Phenols in the Press-juice.

Preliminary experiment.

A 750-cc. portion of press-juice, prepared according to a method developed in this laboratory (26a) was steam distilled until 2 l. of distillate had collected. The distillate was treated with 4 g. of sodium hydroxide, evaporated to about 150 cc., acidified with hydrochloric acid and made up to 200 cc. It was postulated that this solution would contain the free volatile phenols. It gave a positive test with the phenol reagent of Hanke and Koessler (19), the colour being yellow. This reagent was selected in preference to that of Folin and Denis (11) since it is less subject to error through interfering substances.

The residue in the distilling flask was filtered from the coagulated proteins and reduced to a volume of 750 cc. An amount of concentrated hydrochloric acid (18.75 cc.), equivalent to that used in the hydrolysis of combined phenols in urine, was added and the solution was steam distilled until 2 l. of distillate had collected. This solution was concentrated and made up to volume as outlined above; it was postulated that the final solution would contain such volatile phenols as had been previously held in simple combination, possibly as glucosides. This solution also gave a yellow colour with the reagent.

Attempt to develop a quantitative method.

The amount of distillate which would have to be collected in order to obtain all the volatile phenols was first investigated. The distillate was collected in 100-cc. portions.

Issues in interpreting data in terms of the theory of the [illegible]

Preliminary assumptions.

A [illegible] model of [illegible] involves [illegible] [illegible] [illegible]

Each of these was tested with the reagent and the depths of colour developed were compared. The depth of colour decreased slowly until the tenth portion when it remained about constant. Similar results were obtained with the distillate after adding acid. In spite of the fact that this experiment indicated that no definite end-point could be obtained it was felt that a rough quantitative comparison between varieties might be made by this method by distilling over a definite volume.

Before attempting to decide what this volume should be it was necessary to investigate the quantitative aspects of the colorimetric determination. The difficulties were two; to get a standard which would match the colour produced by the unknown phenol in the distillate; and to find the conditions which would give a development of colour, the depth of which would be proportional to the amount of phenol present. Several simple phenols were tried as standards without success. Recourse was then had to the indicators used by Hanke and Koessler (19). A standard containing 2.45 cc. of their phenol red solution and 0.05 cc. of methyl orange solution, made up to 100 cc., was finally adopted. The time required to develop a maximum colour which is proportional to the concentration of phenol present has been found by these workers to vary with different phenols. In many cases the colours were found to develop and fade quickly, leaving only a short time in which accurate determinations could be made. After numerous attempts to solve this problem for the unknown phenols present in the distillate, the reagent had to be discarded as impracticable.

The reagent of Folin and Denis (11) gave promise of better results since it gives the same colour with all phenols, and since the colour does not fade quickly and can be developed under standard conditions for all phenols. After reviewing the literature on the use of this reagent and experimenting with several methods, the technique of Rakestraw (31) was adopted. The reagent was made up according to the improved method due to Wu (52). A fresh portion of the reagent was diluted with three parts of water before using, and 0.5 cc. of this solution were found to be sufficient for the development of maximum colour in 10 cc. of the unknown solution. After adding the reagent to 10 cc. of the solution in a test tube and mixing, 2 cc. of 20% sodium carbonate solution were added. The solution was then allowed to stand until the excess reagent had been destroyed by the carbonate. Rakestraw found ½ min. sufficient, but Chapin (5) found that 20 mins. were required and he adopted a standard time of 30 mins. Under our conditions Chapin's technique was found to be necessary. After allowing the mixture to stand 30 mins., 1 cc. of 5% sodium cyanide solution was added and the test tube was stood in a boiling water bath for 1½ mins., when it was transferred to running water for 3 mins. The colour developed was found to remain constant for at least two hours but colorimetric readings were generally made immediately. Comparisons were made in a Leitz-Buerker colorimeter with a standard solution of resorcinol treated by the same method. Resorcinol was adopted as a standard by Benedict and Theis (4) since it can be weighed out directly. If phenol is used as a standard it must be re-

standardised iodometrically after it has been made up.

The quantitative accuracy of the method was tested by running determinations on various concentrations of the concentrated distillate and also on various concentrations of resorcinol. The results expressed in both cases as resorcinol equivalent in mg. per litre, are reported in Table 1. They show a satisfactory accuracy for the present investigation.

TABLE 1. - ACCURACY OF DETERMINATION WITH THE REAGENT OF FOLIN AND DENIS.

Distillate		Resorcinol	
Determined	Calculated	Determined	Calculated
3.9	3.8	6.9	7.0
3.5	3.4	5.4	5.3
3.0	Standard.	4.1	4.2
2.9	2.7	3.5	Standard.
2.3	2.3	3.3	3.2
1.9	1.9	2.9	2.8
1.6	1.5	2.2	2.1
		1.8	1.8

An accurate method being now available a quantitative comparison of the amount of phenol in successive portions of the distillate was made. For this purpose 350 cc. of the press-juice of Khapli wheat plants was steam-distilled and the

[illegible]

[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]		

[illegible]

distillate collected in 100 cc. portions. The data are reported in Table II. These results show that it would be impracticable to attempt quantitative comparisons by this method since there is no indication of an end-point at which the distillation might reasonably be stopped.

TABLE II. - AMOUNT OF PHENOL IN SUCCESSIVE 100 cc. PORTIONS OF THE STEAM DISTILLATE.

Portion number	Resorcinol equiv.	Portion number	Resorcinol equiv.	Portion number	Resorcinol equiv.
1	6.1	9	2.4	17	1.8
2	3.1	10	1.9	18	1.7
3	3.0	11	2.1	19	1.9
4	2.7	12	2.2	20	1.7
5	2.3	13	2.7	21	1.7
6	2.2	14	1.7	25	2.0
7	2.3	15	1.7	30	1.8
8	2.5	16	1.9		

[illegible] collected in 100 cc. portions. The [illegible] were [illegible] in [illegible] cc. These results are [illegible] in [illegible] as [illegible] conditions [illegible] of [illegible] where there is no indication of [illegible] the [illegible] [illegible] [illegible].

Table 1. - Record of [illegible] in [illegible] cc. [illegible] [illegible] [illegible].

[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

Attempt to Determine Total Phenols by Ether Extraction.

As has been previously pointed out, the problem of determining phenols colorimetrically is mainly that of getting rid of interfering substances. Phenols can be extracted from an acidified solution by ether and extracted from ether by alkaline solutions. These properties have been made use of by various workers (20, 43) for the separation of phenols. An attempt was therefore made to use this method for the extraction of phenols from the press-juice of wheat.

Preliminary experiment.

A 25-cc. portion of press-juice from Khapli was shaken out with 50 cc. of ether. Three layers were formed on centrifuging; at the bottom, a yellow aqueous layer, above this a dense emulsion of the protein material, light green in colour, and on top a redish green ether layer. The top layer was drawn off. The procedure was repeated and the ether extracts were combined. They were then extracted with 25 cc. of 10% sodium hydroxide solution. This extract was bright yellow, it was neutralised with hydrochloric acid and turned a pale brownish yellow. It then gave a deep blue with the Folin-Denis reagent. The ether was extracted a second time with sodium hydroxide solution giving a greenish yellow extract, which turned pale green on neutralising and also gave a blue with the reagent. The green colour of the solution interfered with colorimetric comparisons.

[illegible] of [illegible] [illegible] [illegible]

[illegible] [illegible]

[illegible]

[illegible]

[illegible]

This experiment showed that it would be necessary to get rid of the chlorophyll and proteins in order to make satisfactory extractions and colorimetric determinations. This was effected by precipitation with the tungstic acid reagents of Folin and Wu (13) which have been widely used for clearing blood on which determinations of phenols were to be made. The investigation of this phase of the method is reported in a later section.

Development of quantitative method.

The following method was outlined for the determination of phenols in the press-juice. Add to 25 cc. of press-juice, 5 cc. of 10% sodium tungstic and 5 cc. of 2/3 N. sulfuric acid, shake, centrifuge and decant through a filter. Take a 10-cc. aliquot of the filtrate and extract a sufficient number of times with 20 cc. of ether to ensure quantitative removal of the phenols. Combine the ether extracts and extract with one-tenth the volume of 10% sodium hydroxide, a sufficient number of times to ensure quantitative removal. Neutralise the sodium hydroxide extracts with hydrochloric acid, make up to volume, and determine phenols by the Folin-Denis reagent.

In order to make successive extractions of the 10-cc. portion of filtrate an apparatus similar to that of Hanke and Koessler (20) was used. A special extraction tube having a ground glass stopper and a 10-cc. bulb attached to the bottom by a fairly long neck was devised. (Plate 1). After each extraction sufficient water is added to bring the separation

This experiment showed that it would be necessary to get rid of the [illegible] and [illegible] in order to make satisfactory measurements of [illegible]. Unfortunately this was not attempted in preliminary with the [illegible] [illegible] [illegible]

surface between the aqueous solution and the ether into the neck. This allows of a more accurate separation than could be made if the surface were larger. The ether is drawn off into a separatory funnel by means of suction.

Hanke and Koessler (20), working with solutions of pure phenols, found that ten extractions with twice the volume of ether were required to effect quantitative removal. This point was investigated for our conditions. Eleven successive extractions of 10 cc. of filtrate with 20 cc. of ether were made. Each ether extract was extracted with 2.5 cc. of 10% sodium hydroxide. These alkaline extracts decreased in depth of colour from bright yellow to almost colorless. Each was neutralised with dilute hydrochloric acid and made up to a volume of 25 cc. and its phenol content determined.

Two such series of determinations were made on the press-juice from Khapli plants; the data are reported in Table III. Since the amount of phenol decreases steadily with each extraction, and since the amount in the 11th extract was less than 0.5% of that in the first ten extracts, ten extractions with twice the volume of ether were adopted as a standard number.

TABLE III. - EFFICIENCY OF ETHER EXTRACTION.

Variety - Khapli.

Extraction number	Phenols as resorcinol in mgs.	
1	1.500	1.250
2	0.408	0.350
3	0.145	0.108
4	0.070	0.055
5	0.043	0.030
6	0.040	0.023
7	0.030	0.015
8	0.020	0.013
9	0.013	0.010
10	0.015	0.008
11	0.010	0.008
Total	2.294	1.870
2nd 5 extrns. as % of 1st 5.	5.4%	3.9%
11th extrn. as % of 1st 10.	0.44%	0.43%

The difference between the amount of phenol found in each determination may be accounted for by several factors. The plants used in the second series were ten days older than those used in the first. The total solid content of the juice used in the first determination was 7.5% whereas that of the juice used in the second was 7.2%. Different precipitation techniques were also used. In the first series the juice was

TABLE IIB. - SUMMARY OF MEANS [illegible].

INDEX - [illegible].

[illegible]	[illegible]	[illegible]
1	1.000	1.000
2	0.000	0.000
3	0.347	0.000
4	0.000	0.000
5	0.000	0.000
6	0.000	0.000
7	0.000	0.000
8	0.000	0.000
9	0.000	0.000
10	0.000	0.000
11	0.000	0.000
[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]

brought to the boil for three minutes after adding the reagent; no heat was used in precipitating for the second series.

The number of extractions required to remove the phenol from the ether was also investigated. The combined ether extracts, having a volume of 200 cc., were extracted three times with 10 cc. of 10% sodium hydroxide and these extracts were combined (extract 1.). The ether was then extracted twice more with 5 cc. of sodium hydroxide solution, these were also combined (extract 2). It was found that after neutralising, these solutions would have to be diluted to 10 times their volume in order to prevent the salt concentration from interfering with the colorimetric determination. In only one out of three determinations did sufficient colour develop in extract 2 for quantitative comparison. In this case the amount of phenol in extract 2 was 0.22% of that in extract 1. Three extractions with 10 cc. of 10% sodium hydroxide were therefore adopted as a standard number.

A recapitulation of the method follows. A 25-cc. portion of press-juice was pipetted into a 100-cc. centrifuge tube, 5 cc. of 10% sodium tungstate solution were added followed by 5 cc. of 2/3 N. sulfuric acid. The solution was shaken, centrifuged and decanted through a filter. A 10-cc. aliquot of the filtrate was pipetted into the extraction tube and extracted 10 times with 20-cc. portions of ether. The ether extracts were drawn off into a separatory funnel. The ether was then extracted 3 times with 10 cc. of 10% sodium hydroxide solution. The sodium hydroxide solutions were combined and

made just acid to litmus paper by titrating with dilute hydrochloric acid. The solution was then made up to a volume of 300 cc. in a volumetric flask. An aliquot of this solution was used for colorimetric determination of phenols with the reagent of Folin and Denis.

Volatile and non-volatile phenols were determined on the final solution. A 200-cc. portion was measured out and washed into a 500-cc. distillation flask with 50 cc. of distilled water. When 100 cc. of distillate had collected 50 cc. of water were added through a separatory funnel set in the stopper of the flask. A further 50 cc. was distilled off, 50 cc. of water added and a fourth 50-cc. portion distilled. The solution remaining in the distillation flask was washed back into a 250-cc. volumetric flask and made up to volume. The concentration of phenol in the distillate was too low for determination. The phenol in the remainder was determined and the volatile phenols were obtained by difference.

The method outlined above was then used in making determinations of phenols in the series of eight wheat varieties of graded resistance to rust. The plants were grown in the greenhouse and determinations were made on sixteen-day-old tissue. The results are reported in Table IV.

TABLE IV. - PHENOL CONTENT OF PRESS-JUICE OF WHEATS.

Variety	Phenol as resorcinol: mgs. per 100 g. juice.		
	Total	Non-volatile	Volatile
Khapli	15.6	12.4	3.2
Vernal emmer	9.1	7.8	1.3
Kanred	5.9	4.3	1.6
Kota	10.7	7.7	3.0
Kubanka	16.0	11.0	5.0
Mindum	11.8	7.8	4.0
Marquis	5.9	5.0	0.9
Little Club	5.5	3.6	1.9

Varieties in order of resistance to rust.

One series of determinations is insufficient to establish definite differences between varieties. However since Khapli contained considerably more phenols than any other variety except Kubanka, and since Little Club and Marquis contained the least, the results of this series indicated that the hypothesis that resistant varieties contained more phenols than susceptible varieties, might be substantiated if a sufficient number of determinations were made.

The results of this series of determinations did not confirm those of the previous investigation of the efficiency of the ether extraction. Whereas the earlier investigation showed that an eleventh extract contained less than 0.5% of the amount of phenol in the first ten extractions, throughout

TABLE IV. - [illegible]

Variety	Number of [illegible]: [illegible]		
	Total	[illegible]	Variety
[illegible]	[illegible]	[illegible]	[illegible]
Varnal amber	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]

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[illegible]

the last series of determinations the eleventh extract contained from 1 to 2%. An increase in the number of extractions did not seem advisable since the method was already cumbersome owing to the large number of manipulations. An attempt was therefore made to obtain quantitative extraction by the use of a continuous extraction apparatus similar to that of Palkin, Murray and Watkins, (27).

The apparatus is shown in Plate 1. A 10-cc. portion of the solution to be extracted is placed in the tube T and 75 cc. of ether is boiled in the flask F. The ether vapour passes up the side-neck, condenses and drips down into the funnel A which reaches to the bottom of the tube. By bubbling up through the solution the ether extracts the phenols and passes back into the boiling flask by means of the side-neck.

The use of this apparatus brought out some faults of the method which had not previously been recognised. Some water was carried over into the boiling flask in solution in the ether. After 24 hrs. extraction about 2 cc. of water separated out in the flask. It was bright yellow in colour whereas the ether was very pale yellow. This suggested that the substance responsible for the yellow colour was not sufficiently soluble in ether to be quantitatively extracted, but was in reality being carried over in the water dissolved in the ether.

The filtrate on which extractions were being made had a pH of 2.3. It seemed possible that the compound might be less soluble in, and more easily extracted from, a solution

Plate I. - Extraction apparatus.

which was almost neutral. This point was investigated by running concurrent extractions on two samples of filtrate from Khapli press-juice, the second of which was titrated back with sodium hydroxide to a pH of 6.2 using the potentiometer to obtain an endpoint in order not to add a coloured indicator. The apparatus was run 24 hrs., the boiling flasks were changed and again run 24 hrs., the flasks were again changed and a final run of 48 hrs. was made. During this time water had to be added to the solutions in order to make up for that carried over in the ether. The results are reported in Table V .

TABLE V . - EFFECT OF ACIDITY OF FILTRATE ON RATE OF EXTRACTION.

Extraction.	Phenol as mgs. resorcinol.	
	pH 2.3	pH 6.2
First 24 hrs.	0.90	0.53
Second 24 hrs.	0.21	0.09
Second 48 hrs.	----	0.14

They show that extraction was more efficient with the more acid solution. The fact that the second 48 hrs. extraction contained 22.5% of the amount extracted in the first 48 hrs. is also worth noting since it shows the inefficiency of the extraction.

The relative amount of the yellow compound carried over in the ether per se, and in the water in solution in the ether, was investigated in the following manner. A 25-cc.,

continuous-extraction apparatus was run for 48 hrs. without adding any water. The water which collected in the boiling flask was drawn off in a separatory funnel and its phenol content determined. The ether was divided into two equal portions. One portion was dried with anhydrous sodium sulfate, filtered and the sodium sulfate rinsed with anhydrous ether. Each portion of ether was then extracted with sodium hydroxide and its phenol content was determined in the usual manner. The results of two determinations are reported in Table VI .

TABLE VI . - DISTRIBUTION OF PHENOL BETWEEN ETHER AND WATER.

Fraction	Phenol as mgs. resorcinol.	
Wet ether	0.560	0.380
Dried ether	0.490	0.316
Water	0.215	0.236
Total in dry ether by calc.	0.980	0.632
Total in water by calc.	0.355	0.364

These results seemed to confirm our surmise that the substance responsible for the yellow colour was not sufficiently soluble in ether to make quantitative extraction possible.

Proof that the substance responsible for the yellow colour was almost insoluble in anhydrous ether was obtained in the following manner. A preliminary evaporation of a portion

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[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]

of filtrate in an oven at 60°C. produced a very dark brown waxy residue. The darkening was diagnosed as being due to the increase in acidity on concentration by evaporation. Filtrate to be evaporated was therefore titrated back almost to neutrality. The end-point was determined with a potentiometer in order not to add any coloured indicator. Since the waxy residue did not lend itself to efficient extraction, the following method was used in order to get a powder. The filtrate was absorbed in a ball of glass wool and evaporated. As the residue remained dispersed through the wool, the whole could be ground up to form a powder suitable for extraction.

A portion of filtrate treated in this manner was extracted with anhydrous ether in a Soxhlet apparatus for 48 hrs. The ether was colourless. It was extracted with sodium hydroxide and gave a very pale yellow, almost colourless solution. A little water was then poured through the thimble. The aqueous extract was yellow and turned bright yellow on adding sodium hydroxide solution. The substance responsible for the yellow colour in alkaline solution after extraction of the filtrate by ether was thus shown to be almost insoluble in anhydrous ether. This appeared to explain the failure to obtain quantitative extraction with ether.

This water-soluble yellow pigment was diagnosed from its colour reactions and solubilities, as a compound closely related to the anthoxanthins, or an anthoxanthin glucoside. The anthoxanthins contain phenolic groups and proof that they react with the Folin-Denis phenol reagent was obtained by

testing a solution of pure quercetin.

Since this yellow pigment seemed to be the main phenol present, thus masking the presence of other phenols, if any, and since it could not be extracted quantitatively, the method of ether extraction was dropped pending qualitative investigation of the water-soluble yellow colouring matters present in the wheat plants.

QUALITATIVE ANALYSIS.

The primary object of this analysis was the separation of the water-soluble yellow colouring matters, thought to be anthoxanthins, which had interfered with the quantitative determinations of phenols.

The literature on the extraction, identification, and chemistry of the anthoxanthin pigments of plants is very extensive. The investigations, however, have been confined almost entirely to plants and plant extracts which have been used for dyeing. Perkin and his co-workers carried out much of the work and an excellent summary is available (29).

Anthoxanthins have been shown to occur in two members of the Gramineae: Sando (35) has isolated quercetin from maize, and Thomson (40), an unidentified flavone or flavonol, in both free and glucoside form, from Orchard grass. Turning to other families of crop plants, Sando (36) obtained quercimetrin from sunflowers, and a number of anthoxanthins and related compounds have been isolated from clover flowers (30, 33).

EXPERIMENTAL.

Extraction and precipitation.

Plants of each of the eight wheat varieties of the host series were grown in the field until they were about to shoot. The plants were cut off at ground level and 4 kg. of tissue of each variety was dried at a temperature of about 35°C. The material was allowed to reach equilibrium at room temperature, weighed, and ground to pass a 100-mesh sieve.

Of the different methods which have been used for the preliminary isolation of anthoxanthin pigments from plant tissue, water extraction followed by lead precipitation, was chosen as the most suitable for our conditions. A 400-g. portion of the powdered tissue of each variety was refluxed with 4 l. of water for 2 hr. The mixture was strained through a cloth and the aqueous extract was treated with M/1. lead acetate solution. In order to effect a partial purification, the total amount of acetate solution required was determined by titrating an aliquot of the extract, and one-third of this amount was then added to the extract. A dirty greenish-yellow precipitate came down. It was allowed to settle, the supernatant liquid was decanted off and the precipitate was sucked dry on a buchner funnel. It was found impossible to wash it owing to clogging of the filter paper. The combined filtrate and supernatant liquid was then treated with excess lead acetate solution. A yellow precipitate came down which was separated in the same manner as the above. The supernatant

solution was centrifuged and the material thrown down was combined with the precipitate. The filtrate was then boiled and treated with excess dilute ammonium hydroxide when a further lead precipitate came down. This was also separated as above.

It was hoped that if sufficient pigment were present comparisons of the amounts occuring in different varieties might be made on the basis of the amount of pure pigment extracted. Preliminary investigations showed that this would be impossible. However, since it was thought that the majority of compounds precipitated by lead acetate would be phenolic, it was decided that the lead precipitates should be dried at low temperature and weighed.

The drying was carried out in a small oven, with forced ventilation, at 35°C. for four days, or longer. The weights of the precipitates are reported in Table VII. There appear to be no great differences between varieties in the amounts of substances precipitated as lead salts.

TABLE VII.- WEIGHTS OF LEAD PRECIPITATES FROM WATER EXTRACTS OF WHEAT TISSUE.

Variety	g. ppt. per g. dry tissue.		
	On addition of Pb acet.	On addition of amm. OH.	Total
Khapli	2.76	0.50	3.26
Vernal emmer	----	1.01	----
Kanred	2.92	0.72	3.64
Kota	2.82	0.79	3.61
Kubanka	2.40	0.67	3.07
Mindum	2.56	0.91	3.47
Marquis	2.38	0.47	2.85
Little Club	2.71	0.97	3.68

Varieties in order of resistance to rust.

Decomposition of lead precipitate.

The first lead precipitate and that thrown down by ammonium hydroxide have not yet been analysed. The second lead precipitate was decomposed by suspending it in boiling alcohol and passing in hydrogen sulfide. Since it was treated in several portions, each of which was subjected to a slightly different procedure, a detailed description would be unnecessarily long. In general, 150 g. of the precipitate was suspended in 1500 cc. of boiling 95% alcohol and hydrogen sulfide was passed in for half an hour. The residue was retreated in a similar manner from four to six times. The extracts decreased in colour from reddish-orange, through orange, to pale

yellow. The extracts were combined in various ways and concentrated by distilling off the alcohol in a water bath.

Preparations.

Preparation A.

The first two or three alcoholic solutions, on concentration to about 1/20 of their volume, and after standing in the refrigerator for several days, deposited small white crystals. Most of these formed on the sides and bottom of the beaker. About 0.2 g. of these crystals were obtained after recrystalising once from 95% alcohol. Two compounds were found to be present. These were partially separated by fractional precipitation from 95% alcohol. The crystals were very small. They were examined under the microscope and appeared to be monoclinic needles and tetragonal bipyramids or prism and pyramids. Both these compounds dissolved readily in water, the solution being neutral to litmus. They were not precipitated by lead acetate nor did they react with the phenol reagent of Folin and Denis.

Preparation B.

The later alcoholic extracts, on concentration to about half volume, and after standing in the refrigerator several days, deposited long yellow needles encrusted with smaller yellow crystals. On purification by recrystallisation from 95% alcohol, two compounds were found to be present. One of these has been purified by repeated crystallisation from

[illegible]

[illegible]

[illegible]

[illegible]

[illegible]

[illegible]

alcohol and comes down in long, very pale yellow, transparent glistening monoclinic plates. The other compound has not yet been purified. It crystallises in small yellow tetragonal prism and pyramids. These often aggregate into long chains and feather forms. Both these compounds were insoluble in neutral, acid and alkaline solutions. They were readily soluble in, and recrystallised from, alcohol, acetone and acetic acid. They were not precipitated from alcoholic solution by lead acetate.

Preparation C.

The alcoholic solutions, after separation of preparations A and B, were combined and concentrated to a volume of about 150 cc. A reddish-brown syrup resulted. This was poured into 1500 cc. of ether. The ether was decanted from an insoluble dark-brown viscous syrup. The ether was a cherry-red colour. It was washed three times with 1/3 its volume of water; the colour of the washings being reddish-orange, orange-yellow and yellow, respectively, and the ether changing to a deep reddish-orange. (For treatment of the aqueous extract see under Preparation J.) The ether was extracted with 1/10, and twice with 1/20 its volume of sodium hydroxide solution. The ether was then a very pale yellow colour. The sodium hydroxide extracts were respectively, dark reddish-brown, reddish-brown, and yellow. On neutralizing these extracts with dilute hydrochloric acid a small amount of flocculent precipitate came down. The solution became only slightly lighter in colour. The

precipitate was dissolved in 30% alcohol and cooled in the refrigerator. A mixture of bright-yellow and brownish-yellow precipitate came down. The semi-dry residue weighed 0.5 g. An attempt was made to separate the components of the mixture by dissolving the precipitate in glacial acetic acid and treating with concentrated sulfuric acid. No precipitate resulted. The acetic acid was evaporated off at 35°C., and the residue was dried in a vacuum oven at 98°C. A dark brown residue, weighing approximately 0.1 g. resulted. An attempt was made to acetylate the compounds by heating in a test-tube in a water-bath for 2 hr. with an equal weight of anhydrous sodium acetate and 1 cc. of acetic anhydride. On pouring into water a brown precipitate came down. This was dissolved in 30% alcohol and cooled in the refrigerator. A mixture of brownish yellow and bright yellow precipitate came down. The acetylation apparently failed. The properties of the original pigments, however, had been lost, since the precipitates were no longer soluble in neutral or acid solution, nor were they precipitated from alcoholic solution by lead acetate.

Preparation D.

The neutralised sodium hydroxide extract of the ether, was strongly acidified with hydrochloric acid and extracted three times with twice its volume of ether. The aqueous solution turned from dark reddish-brown to yellow. The ether solution was orange-yellow. It was concentrated to small volume by distillation and finally evaporated to dryness at 35°C. A brown toffee-like residue resulted. This

was treated with several small portions of chloroform. (For treatment of the chloroform extract see under Preparation G.) The insoluble residue was a pale buff-coloured material which was easily powdered. This was dissolved in 95% alcohol and filtered from a few black specks. The alcoholic solution was reddish-brown with a slightly green fluorescence. It was evaporated to dryness at 35°C. The components of this powder were partially separated by dissolving in 50 cc. of acetone and pouring into 250 cc. of petrol ether. A brownish-red syrup separated out at the bottom of the beaker. On standing it solidified into a yellow mass, contaminated with what appeared to be white crystals. This residue was twice retreated with acetone and petrol ether in a similar manner, the syrup being drawn off instead of being allowed to solidify. (For treatment of the acetone and petrol-ether solution see under preparation E.) The syrup was evaporated to dryness; a deep-orange coloured residue contaminated with a lighter-yellow substance remained. The mixture was washed with a little water, leaving a curdy chocolate-brown residue. This was warmed with a little 10% alcohol. The chocolate-brown colour disappeared, leaving a few milligrams of a dark-brown residue. This dissolved in alcohol giving an orange-red solution. We have so far been unable to get anything but amorphous precipitates of this compound from mixtures of alcohol and water. In alcoholic solution it gives a pale yellow flocculent precipitate with lead acetate and reacts strongly with the Folin-Denis reagent.

Preparation E.

The acetone and petrol-ether solution, from the purification of preparation D, deposited a cream-coloured precipitate. This was filtered off and dissolved in water in which it was very soluble. (For treatment of the filtrate see under preparation F.) On evaporation to dryness, there remained a mass of cream-coloured crystals covered by a yellow cream-coloured scale. This residue weighed approximately 0.4 g. We have so far been unable to find any solvent from which to crystallise this compound. In aqueous solution it turns blue litmus red, gives a flocculent white precipitate with lead acetate, and a very faint reaction with the Folin-Denis reagent.

Preparation F.

The filtrate of acetone and petrol ether, after precipitation of preparation E, was evaporated to dryness leaving a few milligrams of impure white crystals. These were dissolved in ether and recrystallised by evaporation. The crystals were very small. Examination under the microscope showed two compounds to be present, one appearing in clusters of fine colourless needles and the other in tetragonal pyramids with base. They were insoluble in neutral, acid and alkaline solution, and were not precipitated from alcoholic solution by lead acetate.

Preparation G.

The orange-red chloroform solution, from the purification of preparation D, was washed twice with an equal volume of

water. The aqueous extracts were respectively bright and pale yellow. (For treatment of these extracts see under preparation H.) The chloroform was evaporated to dryness at 35°C., leaving approximately 3 cc. of a reddish-brown syrup. This was dissolved with difficulty in about 1500 cc. of water. The solution was yellow in colour, acid to litmus, gave no precipitate with lead acetate, but reacted strongly with the Folin-Denis reagent. No crystals of this compound have yet been obtained.

Preparation H.

The aqueous extract from the chloroform was evaporated to dryness at 35°C. leaving a buff-coloured, sticky, toffee-like residue. This was dissolved in acetone and poured into petrol ether. A brown syrup separated; it was evaporated to dryness leaving a waxy residue, which was pale yellow in colour with areas of a darker orange yellow. This residue dissolved readily in water. The solution was reddish-orange in colour, acid to litmus, gave a pale yellow flucculent precipitate with lead acetate, and reacted strongly with the Folin-Denis reagent.

Preparation I.

The acetone and petrol-ether solution, from the purification of preparation H, was evaporated to dryness, leaving a few milligrams of bright yellow crystals covered by a pale yellow wax. The mixture was readily dissolved in water giving a pale yellow solution which was acid to litmus, gave a creamy white precipitate with lead acetate and reacted with the Folin-Denis reagent.

Preparation J.

The water extract of the ether used in the separation of preparation C, was evaporated to dryness at 35°C. leaving a dark shiny brown toffee-like substance. This was dissolved in acetone and diluted with half its volume of petrol ether. A dark syrup separated and was drawn off. The syrup was diluted with 300 cc. of water. A small amount of brown precipitate came down and was filtered off. The filtrate was red in colour. It was boiled with a little carbon black which absorbed a little oil floating on the surface of the solution. The filtrate from the carbon black was treated with excess lead acetate solution. A pale yellow precipitate came down; it was filtered off and washed with hot water. The filtrate was boiled and treated with excess dilute ammonium hydroxide when a further lead precipitate came down. The lead precipitates were combined, mixed into a thin paste with water and poured into 400 cc. of boiling dilute sulfuric acid. After centrifuging, the orange-red solution was decanted from the lead sulfate. The solution was shaken three times with an equal volume of ether. The ether extracts were coloured orange-yellow, yellow and paler-yellow; the solution remained orange-red. It was then shaken once with 100 cc., and twice with 50 cc. of amyl alcohol. This took most of the pigment out of the solution, leaving it yellow. The red amyl alcohol solution was evaporated to dryness on a water bath, leaving a brown tarry residue. This was dissolved in about 50 cc. of alcohol and diluted with 400 cc. of water. The solution changed to a light-brown opaque colour. On centrifuging a

Preparation A.

The water extract of the ether used in the separation of preparation C, was evaporated to dryness at 35°C. leaving a dark shiny brown coffee-like substance. This was dissolved in acetone and diluted with half the volume of petrol ether. A dark syrup separated and was drawn off. The syrup was diluted with 100 cc. of water. A small amount of brown precipitate came down and was filtered off. The filtrate was red in colour. It was boiled with a little carbon black which absorbed a little oil floating on the surface of the solution. The filtrate from the carbon black was treated with excess lead acetate solution. A pale yellow precipitate came down; it was filtered off and washed with hot water. The filtrate was boiled and treated with excess dilute ammonium hydroxide when a further lead precipitate came down. The lead precipitates were combined, mixed into a thin paste with water and poured into 400 cc. of boiling dilute sulfuric acid. After centrifuging, the orange-red solution was decanted from the lead sulfate. The solution was shaken three times with an equal volume of ether. The ether extracts were coloured orange-yellow, yellow and pale-yellow; the solution remained orange-red. It was then shaken once with 100 cc., and twice with 50 cc. of amyl alcohol. This took most of the pigment out of the solution, leaving it yellow. The red amyl alcohol solution was evaporated to dryness on a water bath, leaving a brown tarry residue. This was dissolved in about 50 cc. of acetone and diluted with 100 cc. of water. The solution shaken to a light-brown orange colour. On centrifuging a

chocolate-brown precipitate was thrown down. The properties of the original pigment, however, had been lost. This precipitate was insoluble in neutral and acid solution and was not precipitated from alcoholic solution by lead acetate. It did not react with the Folin-Denis reagent in alcoholic solution.

Outline of the separation of preparations.

Lead precipitate
decomposed by H_2S in boiling alcohol

crystals PREP. A. | crystals PREP. B.

conc. soln.

poured into ether

ether | insoluble

washed with water

water | ether

PREP. J. | extract with NaOH & neutralize

precipitate | filtrate

PREP. C. | acidify, extr. with ether and evap.

extract with chloroform

residue | chloroform

acetone - petrol ether

insol. syrup | solution

PREP. D. | precipitate | solution

PREP. E. | PREP. F.

wash with water

water | chloroform

evaporate to dryness | PREP. G.

acetone - petrol ether.

insol. syrup | solution

PREP. H. | PREP. I.

DISCUSSION.

The complicated procedures by which most of the preparations were separated, and the length of time during which the work lasted, make it unwise to assume that the compounds isolated occur as such in the plant. However, in spite of this limitation, the analysis has provided information which will be of considerable help in outlining further work of both a quantitative and qualitative nature.

At least ten different compounds were extracted from the lead precipitate. Seven of these were obtained in crystalline form, but owing to the extremely small quantities present, only one could be purified. Tests carried out on the crude preparations showed that none of these compounds was phenolic.

Evidence which indicates the presence of three, or possibly four different pigments, was adduced. All of these are phenolic compounds and it would appear that these colouring matters are the main phenolic constituents of the wheat plant, possibly excluding tannins.

The main pigment is that which was present in preparation J, and probably in preparations D and H, and also as an impurity in preparation C. This pigment is quite soluble in neutral, acid and alkaline solutions. When concentrated it gives an orange-red colour, and when dilute and orange-yellow to yellow colour. It is not easily extracted from acid solution by ether, but is readily extracted by amyl alcohol. It is extracted from ether by water and more effectively by sodium

CONCLUSION.

The investigated procedures of water [illegible] are practicable when separated, but the result of [illegible] using both the [illegible] mode, may it appear to [illegible] the separate treatment which is done in the plant. However, if prior to [illegible] it limits, the analysis has problems [illegible] which are of considerable [illegible] to multiple [illegible] [illegible] limits and [illegible] [illegible].

It [illegible] [illegible] [illegible]

hydroxide. It is precipitated from aqueous solution by lead acetate. These properties show that it is phenolic in nature but that it does not belong to the anthoxanthins.

Evidence was obtained of the presence of a small amount of an anthoxanthin pigment in preparation C. The colouring matter of preparation J is not precipitated by lead acetate and this suggests the possibility of its being a decomposition product of some other compound. Preparation I appears to contain a small amount of another yellow colouring matter which is distinguished by its ready solubility in water.

In considering the number of pigments which were shown to be present, it must be remembered that none was isolated in pure form, and that in several cases the pigment was partially destroyed during the course of the extraction. The conclusions drawn have therefore been based largely on observations made during the course of the analysis.

INVESTIGATION OF THE PRECIPITATION OF PROTEINS FROM PRESS-JUICE OF WHEAT BY TUNGSTIC ACID.

INTRODUCTION

In the precipitation of nitrogenous material by the tungstic-acid reagent of Folin and Wu (13) a certain degree of acidity is necessary. In the precipitation from blood the above named investigators added a solution of 10% sodium tungstate and an equal quantity of 2/3 N. sulfuric acid. This gave a slight excess of acid over that required to neutralise the combined alkalinity of the sodium tungstate and the blood serum. Rumsey (34) adapted this method to the precipitation of proteins from cereal extracts. The original technique was not satisfactory owing to the high buffer value of the phosphates and proteins present, which prevented a sufficient increase in the hydrogen-ion concentration. Placing this on a pH basis, he found that optimum precipitation of nitrogen compounds and clarification of the solution were obtained at a pH of 2 or less, which he obtained by adding concentrated sulfuric acid to the end-point of thymol blue. Merrill (25) investiated the precipitation of protein from diphtheria antitoxin serum and from peptone solutions. With the former, the zone of maximum precipitation occurred at pH 5.0 with a slight increase at pH 2.8. With the latter, the precipitation increased considerably as the pH decreased from 4.0 to 1.0, with a slight reversal of precipitation beyond this at the acidity of 10% of acid. It appears therefore that the

INVESTIGATION OF THE PRECIPITATION OF PROTEINS FROM DILUTE SOLUTIONS BY MEANS OF TRICHLOROACETIC ACID.

INTRODUCTION

In the precipitation of microscopic material by the trichloroacetic reagent of Gilvarg and Dennis (1951) a certain degree of acidity is necessary. In the precipitation from blood the above group investigators added a solution of 100 sodium formate and an equal quantity of 6/N. solution (1951). This gave a slight excess of acid over that required to neutralize the chemical alkalinity of the sodium formate and the blood serum. Gomori (1951) applied this method to the precipitation of proteins from cerebral extracts. The original technique was not satisfactory using 50 per cent of the total volume of the phosphates and proteins present, which prevented a sufficient increase in the hydrogen-ion concentration. Starting this on a pH basis, he found that optimum precipitation of nitrogen compounds and clarification of the solution were obtained at a pH of 1 or less, which he obtained by using concentrated solution; said to be sub-zero of thermal kind. Murrill (1952) investigated the precipitation of proteins from dilute acetic antitoxin serum and from neptune equilines. With the former, the zone of maximum precipitation occurred at pH 5.0 with a slight increase at pH 2.0. With the latter, the precipitation increased considerably as the pH decreased from 1.0 to 1.0, with a slight reversal of precipitation beyond this at the acidity of 10% of acid. It appears therefore that the

optimum conditions of acidity for maximum precipitation vary with the nitrogenous compound present. For this reason an investigation was made of the conditions necessary for the maximum precipitation of nitrogenous compounds from the press-juice of wheat.

EXPERIMENTAL

Preliminary investigation.

The original Folin-Wu technique, of adding equal quantities of 10% sodium tungstate and 2/3 N. sulfuric acid, was investigated for our conditions.

A preliminary experiment in which the amount of precipitation produced by various amounts of the reagent was judged by eye, showed maximum precipitation with 5 cc. of each reagent for 25 cc. of juice. The chlorophyll was carried down with the protein leaving a clear yellow acid filtrate. The efficiency of this precipitation was tested out quantitatively by Kjeldahl determinations of total nitrogen in 10 cc. of juice, in the filtrate from 10 cc. of juice after precipitation with 5 cc. of reagents, and after precipitation with 10 cc. of reagents. The data reported in Table VIII show that an increase in the amount of reagents from 5 cc. to 10 cc. resulted in an increased precipitation of only 0.40%. Precipitation with 5 cc. of reagents for 25 cc. of press-juice was therefore adopted as a standard method, since although a greater amount of reagents would result in more efficient precipitation it would give too great a dilution for efficient extraction. This technique was used in

optimum conditions of acidity for maximum precipitation [illegible] with the nitrogenous compound present. But this reason an investigation was made of the conditions necessary for the maximum precipitation of nitrogenous compounds from the presence of sweat.

[illegible]

Preliminary investigation.

The original Folin-Wu technique, [illegible] normal quantities of 100 medium [illegible] and [illegible] solution [illegible], was investigated for our conditions.

A preliminary experiment in which the amount of precipitation produced by various amounts of the reagent was [illegible] by eye, showed maximum precipitation with 5 cc. of each reagent for 20 cc. of juice. The chlorophyll was carried down with the protein leaving a clear yellow acid filtrate. The efficiency of this precipitation was tested out quantitatively by [illegible] determinations of total nitrogen in 10 cc. of juice, in the filtrate from 20 cc. of juice after precipitation with 5 cc. of reagents, and after precipitation with 10 cc. of reagent. The data reported in Table VIII show that an increase in the amount of reagents from 5 cc. to 10 cc. resulted in an increased precipitation of only 0.[illegible]%. Precipitation with 5 cc. of reagents for 20 cc. of press-juice was therefore adopted as a standard method, since although a greater amount of reagents would result in more efficient precipitation it would give too great a dilution for efficient extraction. This technique was used in

TABLE VIII - EFFICIENCY OR PROTEIN PRECIPITATION.

Treatment	mgs. Nitrogen	
Total nitrogen in 10 cc. press-juice.	51.10	
Heat-coaguable N. " " "	31.17	
Total N. in filtrate from 10 cc. press-juice after pptn. with 5 cc. reagents.	11.52	
Nitrogen pptd. by 5 cc. reagents (by diff.)		39.58
Total N. in filtrate from 10 cc. press-juice after pptn. with 10 cc. reagents.	11.36	
Nitrogen pptd. by 10 cc. reagents (by diff.)		39.74
Increase in precipitation %.		0.40%

precipitating proteins from the press-juice on which determinations of phenols were to be made by the ether extraction method.

Further investigations.

Whilst attempting to develop a method for the automatic extraction of the press-juice by ether, a further investigation of the precipitation reaction was undertaken, in order to study the effect of hydrogen-ion concentration, and to develop a technique in which dilution of the press-juice would be reduced to a minimum by adding more concentrated solutions of the reagents.

In a preliminary experiment various amounts of sodium tungstate solution and sulfuric acid were added, and nitrogen was determined on 10-cc. aliquots of the filtrate. Corrections

TABLE XVII. - [illegible]

Treatment	Sum. difference
Total difference in 20 sec. [illegible]	[illegible]
Half-[illegible]	[illegible]
Total 3. In [illegible]	[illegible]
Other half, with 1 sec. [illegible]	[illegible]
Difference with, in 1 sec. [illegible] in 20 sec.	[illegible]
Total 3. In [illegible]	[illegible]
Other half, with 20 sec. [illegible]	[illegible]
Difference with, in 20 sec. [illegible]	[illegible]
Increase in [illegible]	[illegible]

Statistically [illegible] when the [illegible] is [illegible] [illegible] [illegible] to be made by the other [illegible] method.

Further Investigations.

There [illegible] to satisfy a [illegible] for the [illegible] [illegible] of the [illegible] by [illegible], a [illegible] [illegible] of the [illegible] [illegible] and [illegible], in order to [illegible] the [illegible] [illegible], and to [illegible] a [illegible] [illegible] [illegible]

were made for changes in volume due to the addition of various amounts of reagents. The results are reported in Table IX.

TABLE IX. - PRECIPITATION OF PROTEIN FROM PRESS-JUICE OF WHEAT.

50 cc. of press-juice.

15% sodium tungstate	10% H_2SO_4	pH filtrate	Nitrogen in filtrate equiv. to 10 cc. juice.
cc.	cc.	pH	mgs.
0	0.0	5.8	59.5
0	0.2	5.0	25.5
0	0.5	5.5	39.0
0	1.0	4.4	20.0
0	3.0	2.3	15.0
0	5.0	1.4	15.0
0	7.0	1.1	14.9
1	0.2	5.5	46.5
1	2.0	3.5	15.9
1	4.0	1.7	14.5
4	1.1	5.2	24.0
4	2.8	3.0	13.2
4	4.5	1.8	12.4
4	6.2	1.4	13.4
10	2.7	4.6	13.0
10	4.0	3.0	10.8
10	5.3	2.0	11.1
10	6.5	1.6	11.1
16	4.3	4.0	12.5
16	5.2	2.9	10.6
16	6.1	2.2	10.0

The results show that with acid alone the maximum precipitation probably occurs at about pH 3.0. With a given amount of sodium tungstate the zone of maximum precipitation appears to be about pH 2.0. The precipitation increases with increasing concentrat-

sets data for chosen in terms due to the division of values model of "samples". The results are recorded in table IX.

TABLE IX. - Probabilities of getting 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 or 13 on, or more-times.

Sample number	Log $P_{(n)}$	Probability of getting	Percent in Difference equal, or in or, times
n.	c.	(%)	(%).
0	0.0	0.0	0.0
1	0.0	0.0	0.0
2	0.0	0.0	0.0
3	1.1	0.0	0.0
4	0.0	0.0	0.0
5	0.0	0.0	0.0
6	0.0	0.0	0.0
7	0.0	1.1	0.0
8	0.0	0.0	0.0
9	0.0	0.0	0.0
10	0.0	0.0	0.0
11	0.0	0.0	0.0
12	0.0	0.0	0.0
13	0.0	0.0	0.0
14	0.0	0.0	0.0
15	0.0	0.0	0.0
16	0.0	0.0	0.0
17	0.0	0.0	0.0

The results show that with this plane the maximum probability probably occurs at about 14 A.U. This a plane report a walled tumbling the type of maximum probability appears to be about 14 A.U. The probabilities increase with increasing concentrat-

ion of sodium tungstate, but this effect appears to be due to an excess of reagent in the later series, since the increase in precipitation is not proportional to the amount of reagent added. This effect may be due to errors introduced by calculations for change in volume. A further series was therefore run, in which only 10 cc. of juice was used, all of it being filtered and washed with hot water. Total nitrogen was determined on the combined filtrate and washings. The results are reported in Table X. They show certain irregularities, but it would appear that 3 cc. of sodium tungstate is sufficient for the total precipitation of the proteins.

TABLE X. - EFFECT OF DIFFERENT AMOUNTS OF SODIUM TUNGSTATE ON THE PRECIPITATION OF PROTEINS.

10 cc. of press-juice.

15% sodium tungstate	10% H_2SO_4	pH filtrate	Nitrogen in filtrate.
cc.	cc.	pH	mgs.
1	0.92	1.69	11.5
2	1.06	----	10.7
3	1.20	----	10.2
4	1.34	----	10.5
5	1.48	----	10.1
6	1.62	----	10.8
7	1.76	2.73	10.6

The investigation was dropped at this point, since the ether-extraction method for phenols was discarded, and in

consequence the tungstic-acid precipitation was no longer required. However it is hoped that time will be found in the near future to confirm these conclusions by further and more accurate determinations.

SUMMARY.

1. Evidence has been adduced that phenolic compounds may be associated with rust resistance in wheat, and preliminary studies have been carried out on methods of estimating volatile and non-volatile phenolic compounds in wheat plants.

2. A tentative method has been developed for determining phenolic compounds of the press-juice from wheat-leaves, by ether extraction of the filtrate after precipitation of the proteins and chlorophyll by tungstic acid. The phenolic compounds are then extracted from the ether by sodium hydroxide solution and determined in the neutralized solution by the reagent of Folin and Denis.

3. A single series of determinations of phenolic compounds, on a series of eight wheat varieties of graded resistance to rust, by the above method, indicated that resistant varieties contain greater amounts of phenolic compounds.

4. The development of the method has been dropped pending an investigation of certain yellow colouring matters of phenolic nature, which are not quantitatively extracted from acid solution by ether, and which thus interfere with the determination.

5. A qualitative analysis has been made on 3200 g. of dry wheat tissue in order to isolate the pigments and other phenolic compounds present. These compounds occur in very

SUMMARY.

1. Evidence has been adduced that phenolic compounds may be associated with rust resistance in wheat, and preliminary studies have been carried out on methods of estimating volatile and non-volatile phenolic compounds in wheat plants.

2. A tentative method has been developed for determining phenolic compounds of the press-juice from wheat leaves, by other extractions of the filtrate after precipitation of the phenolics and colorimetrically by Ingratia unit. The phenolic compounds are then extracted from the wheat by adding aparatic solution and determined in the conventional solution by the reagent of Folin and Denis.

3. A single series of determinations of phenolic compounds, on a series of eight wheat varieties of graded resistance to rust, by the above method, indicates that resistant varieties contain greater amounts of phenolic compounds.

4. The development of the method has been dropped pending an investigation of certain pallid colorimetric matters of phenolic nature, which are not quantitatively extracted from acid solution by ether, and which thus interfere with the determination.

5. A qualitative analysis has been made on that n. of dry wheat plants in order to isolate the pigments and other phenolic compounds present. These compounds occur in very

small quantity and are difficult to separate in pure form.

6. It is believed that three different pigments have been shown to be present. None of these has been obtained in pure form.

7. Seven other compounds have been obtained in crystalline form, but in such small quantities that only one has been purified. These compounds are not phenolic.

8. An analysis of a much larger amount of wheat tissue is projected, with the object of isolating sufficient quantities of the pigments in pure form, for the determination of their properties and for their identification.

9. A preliminary investigation of the precipitation of proteins from the press-juice of wheat by tungstic acid is reported.

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